Systemic Administration of a Conditionally Replicating Adenovirus, Targeted to Angiogenesis, Reduced Lung Metastases Burden in Cotton Rats

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Abstract

Purpose: Angiogenesis is an essential process for solid tumor development. To interfere with angiogenesis, AdPPE3x-E1, an adenovirus that is transcriptionally targeted to replicate in angiogenic endothelial cells, was constructed, by replacing the E1 promoter with the modified preproendothelin-1 promoter, PPE-1-3x, previously shown to induce specific transcription in angiogenic endothelial cells.

Experimental Design: The specificity of AdPPE3x-E1 to endothelial cells was shown by quantitative PCR and immunostaining, and its antiangiogenic effect was evaluated in Matrigel models. The *in vivo* efficacy of AdPPE3x-E1 was also tested in a cotton rat lung metastases model.

Results: The replication rate of AdPPE3x-E1 in endothelial cells was similar to that of AdCMV-E1, a nonselective replicating adenovector, but the replication rate was reduced up to 60-fold in nonendothelial cells. Moreover, AdPPE3x-E1 reduced endothelial cell viability by 90% whereas nonendothelial cells were not affected. In *in vitro* and *in vivo* Matrigel models, endothelial cells infected with AdPPE3x-E1 did not develop capillary-like structures. The systemic administration of AdPPE3x-E1 reduced the lung metastases burden in a cotton rat model by 55%, compared with saline-treated rats, without significant evidence of toxicity. Quantitative PCR analysis showed that the viral copy number of AdPPE3x-E1 was increased 3-fold in the lung metastases but not in the liver, compared with a nonreplicating adenovector control.

Conclusions: We have shown here for the first time an antimetastatic effect induced by an angiogenesis-transcriptionally targeted adenovirus following systemic administration. Because adenovirus replication is more efficient in humans than in cotton rats, we assume a significant effect for AdPPE3x-E1 treatment in fighting human solid tumors and metastases.

Conditionally replicating viruses replicate specifically in selected tissues or pathologic states. The use of conditionally replicating viruses in cancer treatment is known as oncolytic virotherapy. The major attribute that all oncolytic viruses must have is tumor selectivity in terms of gene expression, replication, and cell lysis. One of the methods used to create conditionally replicating viruses is subcloning viral regulatory genes under the control of tumor/tissue-specific promoters, enabling tumor selective expression of viral genes.

Adenoviruses are frequently used in the engineering of conditionally replicating viruses. The E1A region in the adenoviral genome is important for blocking apoptosis and inducing viral DNA synthesis. The expression of this region and other important regions in the viral genome was previously engineered to be regulated by specific promoters, such as the prostate-specific antigen promoter for prostate cancer (1), uroplakin promoter for bladder cancer (2), and surfactant protein B promoter for lung cancer (3).

One of the possible targets for conditionally replicating viruses as oncolytic agents is the tumor vasculature. A solid tumor depends on the host vasculature in the primary growth phase, but soon reaches the size limit beyond which the tumor begins to suffer hypoxia, which results in the induction of angiogenesis, the formation of new blood vessels. The properties of angiogenesis make it an attractive goal for anticancer therapy: targeting the tumor endothelium instead of the tumor itself should enable the drug of choice to spread in the tumor via its blood supply. Furthermore, as the target cell is relatively genetically stable, therapy resistance due to somatic mutations is not anticipated. Other advantages of endothelial targeting include easy reach of target tissue upon i.v. administration and the amplification effect on tumor killing,

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Received 6/29/08; revised 11/11/08; accepted 11/13/08.

Grant support: Israel Cancer Association through the donation from Drs. Daisy and John Merey in honor of their grandchildren Mikala, Alec, and Peter.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-08-1670

Translational Relevance

AdPPE3x-E1 is an adenovirus that is transcriptionally targeted to replicate in angiogenic endothelial cells. Targeting the tumor endothelium should enable the drug of choice to spread in the tumor via its blood supply. Thus, systemic administration could be feasible and allow the reach of hidden metastases. Indeed, the systemic administration of AdPPE3x-E1 reduced the lung metastases burden in a cotton rat model by 55%, compared with saline-treated rats, without significant evidence of toxicity.

Furthermore, AdPPE3x-E1 could be an important adjunct to current cancer therapies that are targeted towards the tumor cells. AdPPE3x-E1 could also help antiangiogenic drugs because it is not an inhibitor of angiogenesis, like avastin, but rather a vector that is aimed to replicate and destroy existing angiogenic blood vessels.

Finally, because adenovirus replication is about 100 times more efficient in humans than in cotton rats, we assume a significant effect for AdPPE3x-E1 treatment in the clinical setting.

because one endothelial cell is known to support the nutritional needs of approximately 100 tumor cells (4).

The angiogenic endothelium differs from the normal vasculature not only in the expression of membrane-associated receptors, adhesion molecules, and integrins (5), but also in the high proportion of proliferating cells (6). These differences enable the design of vectors that selectively target the angiogenic vasculature through transductional or transcriptional targeting. The latter could be achieved by using natural promoters that are preferentially active in angiogenesis-associated endothelial cells. Promoters that are up-regulated in angiogenesis include vascular endothelial growth factor or its receptor (KDR; ref. 7), transforming growth factor-β binding protein (endoglin/CD105; ref. 8), and endothelin-1 (9, 10).

Endothelin-1 (ET-1), a peptide composed of 21 amino acids that is synthesized primarily by endothelial cells, is one of the most powerful vasoconstrictors known (11). It is also a chemoattractant and a mitogen for endothelial cells (12, 13), smooth muscle cells, and tumor cells (14, 15).

ET-1 is derived from preproendothelin-1 (PPE-1). The *PPE-1* gene contains a promoter with endothelial specificity (9, 16). In transgenic mice that express luciferase under the murine PPE-1 promoter regulation, the maximal luciferase expression was found in the aorta (9). The ET-1 promoter contains several transcription factors binding motifs, NF-1, GATA-2, AP-1, and HRE (17, 18), which make it sensitive to hypoxia and other growth factors (19).

We previously showed that a replication-deficient adenovirus vector expressing the *Fas-c* gene, a chimeric death receptor gene containing the extracellular portion of tumor necrosis factor 1 receptor and the transmembrane and intracellular portion of Fas, under the control of the modified murine PPE-1 (PPE3x) showed efficacy in *in vivo* mouse tumor models, reducing lung metastases burden by 60% when compared with saline control, without any evidence of toxicity (19).

In the present research, to improve the effect of the replication-deficient adenovector, we constructed a novel conditionally replicating adenovirus (CRAd), AdPPE3x-E1, targeted to tumor angiogenesis, by replacing the native promoter of the *E1* gene with the modified preproendothelin-1 promoter (PPE3x). The selectivity of AdPPE3x-E1 to replicate, spread, and lyse angiogenic endothelial cells was tested *in vitro* and *in vivo*.

CRAds have been evaluated *in vivo* primarily in immunodeficient mice bearing human tumor xenografts (20, 21). This approach is based on the general belief that human adenoviruses replicate only in human cells and, therefore, cannot be tested in a syngeneic animal model. These mice are immunodeficient and nonpermissive for adenoviruses. Therefore, this model cannot adequately address the effect of the host immune system on the vector-infected tumor or the toxicity of the vector in normal tissues. Moreover, this model cannot address the effect of antiangiogenesis CRAds, because the endothelial cells lining the blood vessels are of mouse origin, and therefore, the CRAds would not be able to replicate in these endothelial cells. To circumvent these shortcomings, a cotton rat tumor model was developed (22).

The cotton rat is an established model for studying the pathology of adenoviruses and other viruses (23–25). In particular, adenovirus serotype 5 (Ad5) has been shown to replicate in the lungs of cotton rats and cause pathology resembling that seen in Ad5-infected humans (26–29). This animal is, at least partially, a permissive host for adenoviral (Ad) replication. Thus, the cotton rat should enable the study of CRAds targeted to angiogenesis, and specifically to lung metastases, as the PPE13x promoter was previously shown to be highly active in this milieu (30, 31).

Toth et al. showed that a cell line (LCRT) of cotton rat origin (22) that was isolated from a spontaneously arisen sarcomatous tumor supported human adenoviral replication. After s.c. injection into cotton rats, these cells formed tumors that were suppressed after intratumoral injection of an oncolytic adenoviral vector. Interestingly, it was observed that the s.c. tumors often metastasized to the lung. Thus, to test the efficacy of an antiangiogenic CRAd, we established a lung metastases model in cotton rats, based on the tumor model previously developed by Toth et al. (22), and tested the effect of AdPPE3x-E1 on these metastases.

Materials and Methods

Cells and cell culture. Normal skin fibroblasts (NSF) were cultured from the arm skin of healthy donors. HepG2 (human hepatocytes) were kindly provided by Prof. O. Stein, Hebrew University Medical School, Jerusalem. These cells were grown in 1 g/L glucose DMEM supplemented with 10% FCS. During the transduction experiments, the medium was replaced by low glucose-DMEM with 2% FCS.

Human embryonic kidney cells (HEK293) were purchased from the American Type Culture Collection. Passages 20 to 26 of 293 cells were used. LCRT cells (22) were kindly provided by Gregory A. Prince, Virion Systems, Rockville, Maryland. These cells were grown in 4.5 g/L glucose DMEM supplemented with 10% FCS.

Human umbilical vein endothelial cells (HUVEC) were produced by collagenase digestion of human umbilical veins as previously described (32). These cells were grown in the EGM-2 Bullet Kit (Cambrex). All medium ingredients were purchased from Biological Industries unless stated otherwise.

Viruses and viral techniques. The replication-competent AdPPE3x-E1, including the adenoviral E1A region under the control of the modified murine PPE1 promoter, was constructed using the "AdEasy" method (Stratagene). The shuttle plasmid that was used as the backbone for homologous recombination was based on the pMK-E1 (33), kindly provided by Dr. Yossef Haviv, Hadassa Ein-Karem, Jerusalem, Israel. Briefly, the shuttle vector was linearized with *Pme*I digestion and subsequently cotransfected into *Escherichia coli* BJ5183 with pAdEasy-1 adenoviral backbone plasmid. The recombinants were linearized with *Pac*I digestion and transfected into the E1-transcomplementing 293 cell line to generate AdPPE3x-E1. The replication competent AdPPE3x-E1 was propagated in 293 cells and purified by double CsCl density centrifugation. Two vectors were used as a control. AdCMV-E1 (33) containing the *E1* gene controlled by the early cytomegalovirus (CMV) promoter was kindly provided by Dr. Yossef Haviv, Hadassa Ein-Karem, Jerusalem, Israel. The vector AdPPE3x-GFP, developed previously (30), contains the green fluorescent protein (GFP) gene controlled by the modified murine PPE-1 promoter.

Assessment of viral DNA replication. Cells were plated at a density of 5×10^5 cells per well in a 6-well plate. Twenty hours later, the cells were infected with the different adenovectors at 1 multiplicity of infection (MOI). The multiplicity of infection was calculated as the number of viruses per target cell. Cells and media were harvested at the indicated time points over 72 h after infection and subjected to quantitative real-time PCR analysis. Genomic DNA was isolated using a Qiamp Kit (Qiagen), following the manufacturer's instructions. The concentration of isolated DNA was determined by spectophotometry. The sequences of the forward and the reverse *E4* primers were GGAGTGCGCCGAGACAAC (anneals between residues 816 and 833 of the E4 orf6 open reading frame) and ACTACGTCCGGCGTTCCAT (anneals between residues 883 and 865), respectively. The sequence of the TaqMan probe was TGGCATGACACTACGACCAACACGATCT (anneals between residues 836 and 863). With optimized concentration of primers and probe, the components of real-time PCR mixture were designed to result in a master mix with a final volume of 20 μL/reaction containing 1× Universal PCR Master Mix (Applied Biosystems), 500 nmol/L forward primer, 500 nmol/L reverse primer, and 250 nmol/L probe. For the assay, 50 ng of extracted DNA sample were added to the PCR mixture in each well. A no-template control received 20 μL of reaction mixture with 1 μL of water. All PCR reactions were done using the Applied Biosystems 7900HT prism real-time PCR instrument (Taqman; Perkin-Elmer/Applied Biosystems). The thermal cycling conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Antihexon staining. Cells were plated at a density of 1×10^5 cells per well in a 24-well plate. Twenty hours later, cells were infected with AdCMV-E1 or AdPPE3x-E1 at 1 MOI and then immunohistochemically stained for viral hexon at 48 and 96 h postinfection, using a Quicktiter Adenovirus titer immunoassay kit (Cell Biolabs).

Cytotoxicity assay. HUVEC, and nonendothelial cells NSF, HepG2, and LCRT were infected with AdCMV-E1, AdPPE3x-E1, or AdPPE3x-GFP at 1, 10, and 100 MOI. Cell viability was assessed 7 d postinfection via the CellTiter 96 AQueous cell viability MTS assay (Promega Gmbh). Briefly, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution was added to the medium at a volume ratio of 1:5, 1 h before termination of incubation. The absorbance was then measured by a spectrophotometer at 490 nm. Cell viability was calculated as a percentage of the viability of the mock-infected cells.

In vitro ***angiogenesis assay.*** Matrigel basement matrix (Becton Dickinson) was diluted 1:2 in cold DMEM. Approximately 200 μL diluted Matrigel were plated into 24-well plates and allowed to gel for 30 min at 37°C before cell seeding. HUVEC were plated on 6-well plates at a density of 5×10^5 cells/well 24 h before infection and infected at 10 MOI with AdCMV-E1, AdPPE3x-E1, or AdPPE3x-GFP. Twenty-four hours after infection, the cells were collected with trypsin/EDTA, resuspended in endothelial growth medium 2, and plated at a density of 5×10^4 cells/well on 24-well plates precoated with Matrigel as described above. After 8 h of incubation, the plates were photographed using a light microscope or a fluorescence microscope, and quantitated by defining capillary tubes as cellular extensions linking cell masses or branch points (34).

In vivo ***angiogenesis assay.*** In all experiments, 9- to 10-week-old female cotton rats (*Sigmodon hispidus*) purchased from Harlan were used. AdPPE3x-E1, AdCMV-E1, or AdPPE3x-GFP (1×10^9 viral particles) was resuspended in 0.5 mL Matrigel supplemented with 200 ng/mL bovine basic fibroblast growth factor. Subsequently, cotton rats were injected s.c., near the abdominal midline, with 0.5 mL Matrigel containing AdPPE3x-E1, AdCMV-E1, or AdPPE3x-GFP ($n = 4$). Additional animals were injected with uninfected Matrigel (saline). Fourteen days after the Matrigel injection, the Matrigel plugs were removed and fixed in PBS-buffered 4% formalin. The angiogenic response was evaluated in von Willebrand factor (VWF)-stained histologic sections for each animal. Formalin-fixed, paraffin-embedded plugs were heated and then deparaffinized using xylene and declining grades of ethanol before being rehydrated in 0.1% Triton X-100 for 10 min. Immunohistochemistry was done using the Blood Vessel Staining Kit (Chemicon) according to the manufacturer's instructions. Microvessel density was quantified by counting the number of microvessels per 400 ×high-power fields over six randomly selected fields. A microvessel was defined as a discrete VWF-positive cluster or single cell adjacent to a lumen.

In vivo ***toxicity assay.*** Cotton rats ($n = 3$, for each time point) were i.v. administered with 1×10^{11} vps of AdPPE3x-E1, AdCMV-E1, AdPPE3x-GFP, or saline. Animals were sacrificed on day 0 (4 h post-i.v. administration), 6, or 14 following injection. The animal's weight was measured every other day for 14 d following i.v. injection. The following parameters were measured on days 0, 6, and 14 post-i.v. injection: plasma urea, creatinine, uric acid, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase, albumin, alkaline phosphotase, and H&E staining of paraffin-embedded liver tissues.

Lung metastases model in cotton rats. Female cotton rats (9-10 wk old) were anesthetized briefly by isoflurane inhalation, and 1.5×10^6 LCRT cells (in a volume of 100 μL saline) were injected s.c. into the flanks. Three cotton rats were injected with saline and served as control. On days 10, 18, 25, and 28 following injection, 2, 3, 3, and 7 rats were sacrificed, respectively. Lung metastasis weight was measured after dissection at necropsy time. Histopathologic analysis of lung tissues was made under H&E-stained paraffin-embedded lung tissues.

To test the effect of AdPPE3x-E1 on lung metastases, 15 d after s.c. injection of LCRT cells into the flanks of female cotton rats (9-10 weeks old), the rats were randomly assigned to three groups ($n = 8$) and anesthetized by isoflurane inhalation followed by ketamine-xylasine i.m. injection. Approximately 6×10^{10} vp of AdPPE3x-E1 or AdPPE3x-GFP in 100 μL saline, or saline (control), were injected systemically into the left ventricle of the heart. Rats were monitored daily for weight and well-being. Once two of the control, saline-injected rats died of metastasis (day 23), all rats were sacrificed. Lung weight was measured on the day of sacrifice. Lung and liver tissues were snap-frozen and stored in cryotubes at −80°C for quantitative real-time PCR for viral genome.

Statistical analysis. ANOVA was done for the parametric variables and Kruskal-Wallis for the nonparametric variables to compare more than two groups in *in vitro* and *in vivo* experiments. Student's *t*-test for the parametric variables and the Mann-Whitney test for the nonparametric variables were done to compare two unpaired groups in *in vitro* and *in vivo* experiments. The Tukey multiple comparison test was conducted when the ANOVA test returned a positive result.

Results

AdPPE3x-E1 replicates preferentially in endothelial cells. To exploit the cell specificity of the PPE1 promoter, we constructed

a recombinant adenovirus (AdPPE3x-E1) in which the native E1 promoter is replaced with the PPE13x promoter. The vector AdCMV-E1, in which E1 expression is controlled by the CMV promoter, served as a positive control. The CMV promoter induces transcription ubiquitiously, with no specificity to endothelial cells. These viruses are deleted in the E3 region extending from 28130 to 30820 Ad5 nucleotides and the E1A promoter region extending from 342 to 488 Ad5 nucleotides. The replication-deficient vector AdPPE3x-GFP (30) encodes the *GFP* gene under the control of the PPE1-3x promoter, and served as a replication-deficient adenovirus control (Supplementary Fig. S1).

To evaluate whether AdPPE3x-E1 specifically replicates in endothelial cells, HUVEC, and nonendothelial cells NSF, HepG2, and LCRT were infected with AdCMV-E1, AdPPE3x-E1, or AdPPE3x-GFP at 1 MOI. The viral copy number was

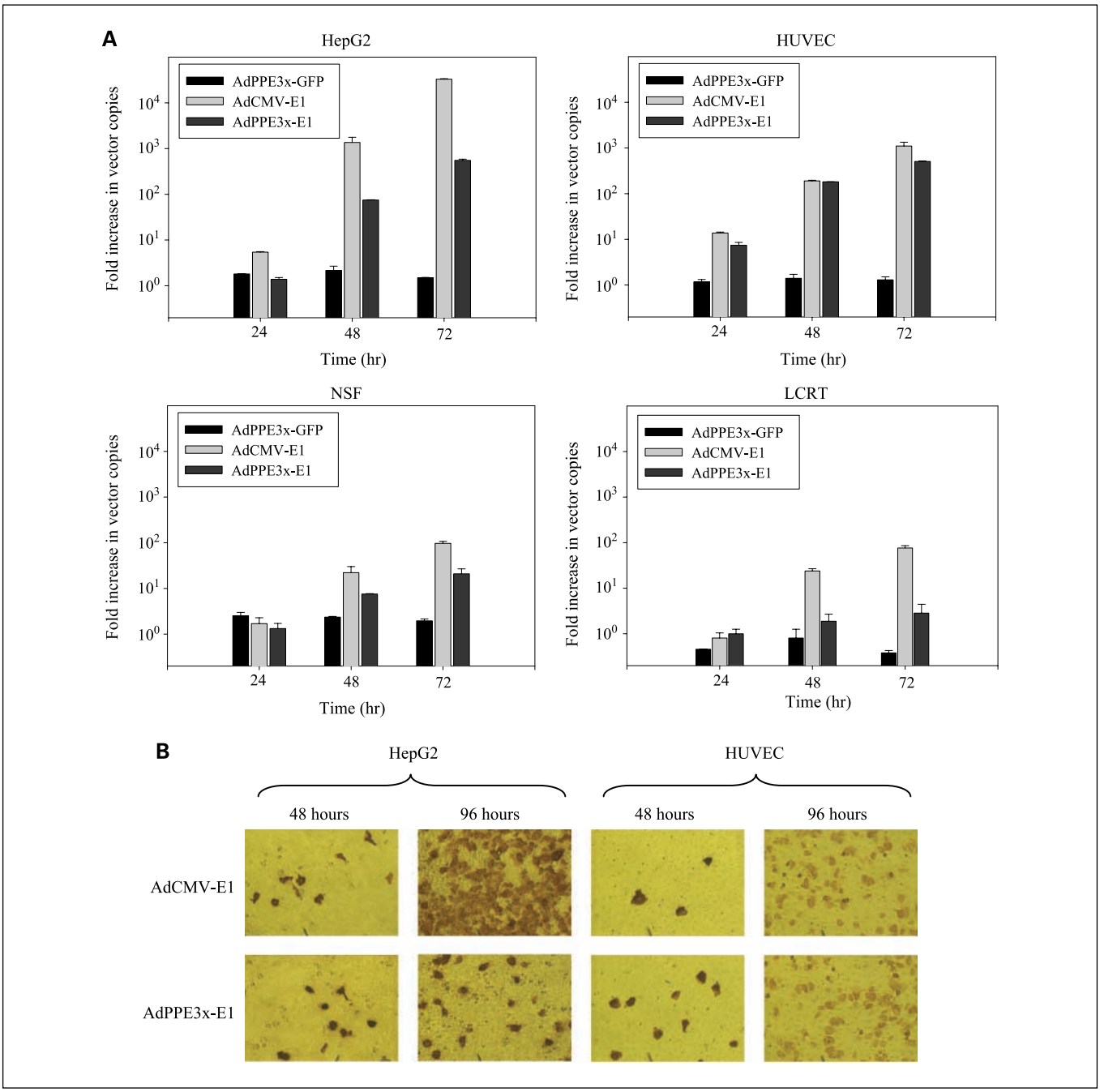

Fig. 1. AdPPE3x-E1 replicates preferentially in endothelial cells. *A,* quantization of adenovirus progeny production in HepG2, HUVEC, NSF, and LCRT cells by quantitative real-time PCR. Cell monolayers were infected at a MOI of 1 with AdPPE3x-E1, AdCMV-E1, and AdPPE3x-GFP adenovirus. The quantity of virus recovered from the cells and medium was assayed 2, 24, 48, and 72 h after infection by quantitative real-time PCR. The fold increase in adenovirus copy numbers is shown compared with the first measurement (2 h). *B,* HepG2 and HUVEC cells cultured in 24-well plates were infected with AdPPE3x-E1 or AdCMV-E1 at a MOI of 1. Cells immunostained with goat antihexon antibody to monitor the replication of viruses 48 and 96 h after infection are shown. Virus spread was assessed with light microscopy, and the darker color corresponds to the adenovirus hexon. Original magnification ×100.

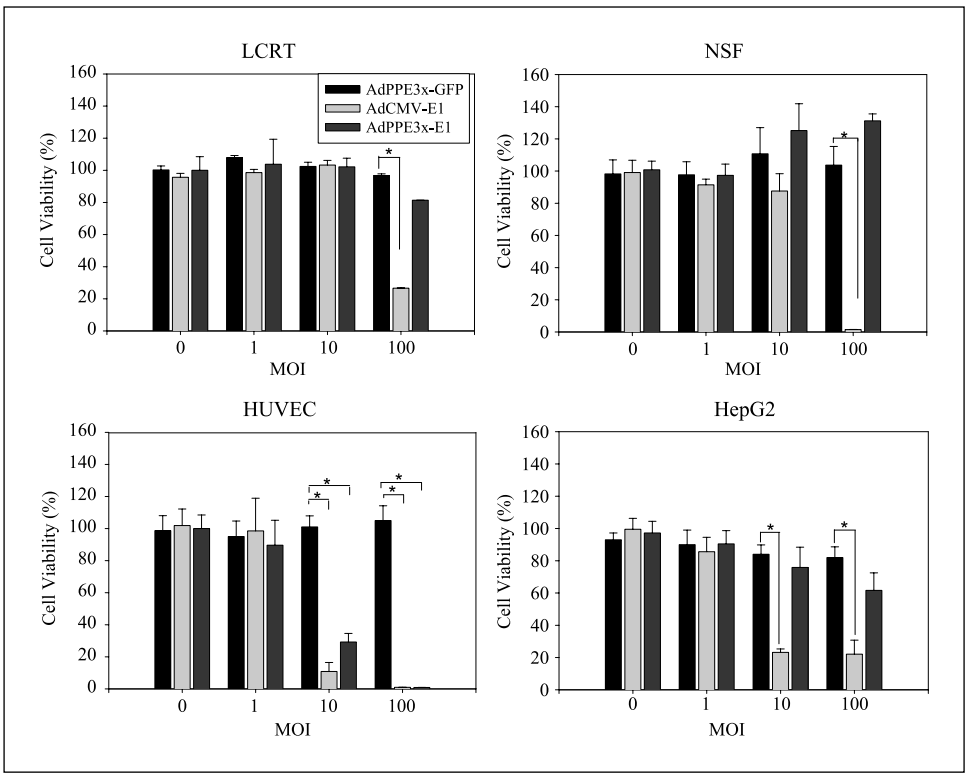

Fig. 2. AdPPE3x-E1 induced cytopathic effect preferentially in endothelial cells. Monolayers of HUVEC, LCRT, HepG2, and NSF in 96-well plates were infected at the indicated MOIs. Cells infected with AdCMV-E1 (*light gray bars*) and E1-deleted AdPPE3x-GFP (*black bars*) viruses and uninfected cells (MOI = 0) were used as controls for AdPPE3x-E1 (*dark gray bars*). Cell viability was assessed quantitatively with MTS assay 7 d following infection. Values are mean ± SE of triplicates. $^*P < 0.01$.

determined by quantitative real-time PCR analysis for the *E4* gene. *E4* is present in all three vectors. AdCMV-E1 and AdPPE3x-E1 replicated in all the cell lines that were tested, whereas AdPPE3x-GFP, the replication-deficient negative control, did not replicate (Fig. 1A).

In HUVEC, AdPPE3x-E1 replicated at levels comparable with those of AdCMV-E1 (Fig. 1A). In nonendothelial cells, however, AdCMV-E1 replicated faster than AdPPE3x-E1. Differences in the rates of viral replication between the different cell lines were quite evident, as, for example, AdCMV-E1 reached a level of $>10^4$-fold increase in viral copy number in HepG2 cells 72 hours following infection. In HUVEC, however, AdCMV-E1 reached a level of $<10^3$-fold increase in viral copy number. According to the formula suggested by Alemany et al. [ref. 35; (AdPPE3x-E1 increase in viral copy number in endothelial cells/AdPPE3x-E1 increase in viral copy number in HepG2) × (AdCMV-E1 increase in viral copy number in HepG2/AdCMV-E1 increase in viral copy number in endothelial cells)], AdPPE3x-E1 is 60-fold more selective to endothelial cells than AdCMV-E1 at 72 hours following infection.

Similar results were observed when viral hexon immunohistochemistry was tested. Viral hexon was detectable in HUVEC and HepG2 cells infected with AdPPE3x-E1 and AdCMV-E1 in a time-dependent manner. However, the number of positively stained cells for viral hexon after HepG2 infection with AdCMV-E1 was higher than that after AdPPE3x-E1 infection (Fig. 1B). In contrast, the number of HUVEC cells positively stained for viral hexon after infection with AdPPE3x-E1 was approximately similar to that after infection with AdCMV-E1. The results show that AdPPE3x-E1 replicates preferentially in endothelial cells, compared with AdCMV-E1.

AdPPE3x-E1 induced cytopathic effect preferentially in endothelial cells. Replication of adenoviruses in the host cell eventually results in host cell lysis. Cells were infected with AdCMV-E1, AdPPE3x-E1, or AdPPE3x-GFP at 1, 10, or 100 MOI. Cell viability was assessed 7 days postinfection via the quantitative MTS assay (Fig. 2).

At 7 days postinfection, HUVEC infected with AdPPE3x-E1 and AdCMV-E1 showed a marked reduction in cell viability, with an 80% to 100% reduction in cell viability in MOI of 100 and 10, compared with cells transduced with E1-deleted AdPPE3x-GFP. Thus, in endothelial cells, the two CRAds had similar effect. In contrast, a clear difference between the cytotoxicity of AdCMV-E1 and AdPPE3x-E1 was observed in nonendothelial cells. AdCMV-E1 induced a 70% to 95% reduction in cell viability ($P < 0.01$) in NSF, HepG2, and LCRT cells in MOI of 100, 10, and 100, respectively, whereas AdPPE3x-E1 caused only minimal cytotoxicity (Fig. 2). These results show that AdPPE3x-E1 infection leads to a selective cytopathic effect in endothelial cells, whereas AdCMV-E1 leads to a nonselective cytopathic effect.

AdPPE3x-E1 infection inhibited HUVEC assembly into capillary-like structures and* in vivo *angiogenesis in a Matrigel model. To test the effect of AdPPE3x-E1 infection on angiogenesis *in vitro* and *in vivo*, a Matrigel model was used. Endothelial cells create intricate spiderweb-like networks on Matrigel-coated surfaces, which are highly suggestive of the microvascular capillary systems and are used as an assay for angiogenesis studies *in vitro* and *in vivo* (36, 37).

HUVEC were infected at 10 MOI with AdCMV-E1, AdPPE3x-E1, or AdPPE3x-GFP. Twenty-four hours after infection, the cells were plated on plates precoated with Matrigel. After 8 hours of incubation, the plates were photographed and quantitated. Capillary tubes were defined as cellular extensions linking cell masses or branch points.

HUVEC infected with AdCMV-E1 and AdPPE3x-GFP, as well as uninfected cells, formed a network of capillary-like

structures. In contrast, HUVEC infected with AdPPE3x-E1 failed to differentiate into capillary-like structures (Fig. 3A). The number of capillary-like structures was 92% and 95% less ($P < 0.01$) in cells infected with AdPPE3x-E1, when compared with cells infected with AdCMV-E1 or AdPPE3x-GFP, respectively (Fig. 3B). A 37% reduction in capillary-like structure formation was also noted in cells infected with AdCMV-E1, when compared with cells infected with AdPPE3x-GFP ($P < 0.05$). The coinfection with 1 MOI of AdPPE3x-GFP and AdPPE3x-E1 showed that the cells were infected with the viruses (Fig. 3C), without apparent capillary-like structure formation in the AdPPE3x-E1-infected cells.

The Matrigel model was also used to test the effect of AdPPE3x-E1 on angiogenesis *in vivo*. Fourteen days after bovine basic fibroblast growth factor–supplemented Matrigel, resuspended with saline, was injected into the flanks of cotton rats, cell infiltration and capillary tube formation were observed. The addition of AdCMV-E1 or AdPPE3x-GFP to the Matrigel did not affect the process of cell invasion within the Matrigel. In contrast, minimal cell invasion and capillary tube formation were observed in the Matrigel plugs resuspended with AdPPE3x-E1 (Fig. 3D). The histologic analysis showed that AdPPE3x-E1 treatment reduced microvascular density in the plug compared with other groups ($P < 0.05$). These results show that infection with AdPPE3x-E1 efficiently inhibits angiogenesis *in vitro* and *in vivo*.

Systemic administration of AdPPE3x-E1 inhibited lung metastases growth. To test the effect of AdPPE3x-E1 on metastasis, we first established a lung metastasis model in cotton rats. The establishment of LCRT cell-line and s.c. primary tumors in cotton rats has been previously described. It was observed that the LCRT s.c. tumors often metastasized to the lung (22). To characterize the induction of lung metastases, LCRT cells were injected s.c. into the flanks of cotton rats, and the lungs were analyzed by gross morphologic and histopathologic analysis.

Lung metastasis weight increased from 0.4 ± 0 g at 10 days to 0.47 ± 0.07 g at 18 days, 0.97 ± 0.12 g at 25 days, and 1.04 ± 0.09 g at 28 days, and was significant at 25 and 28 days when

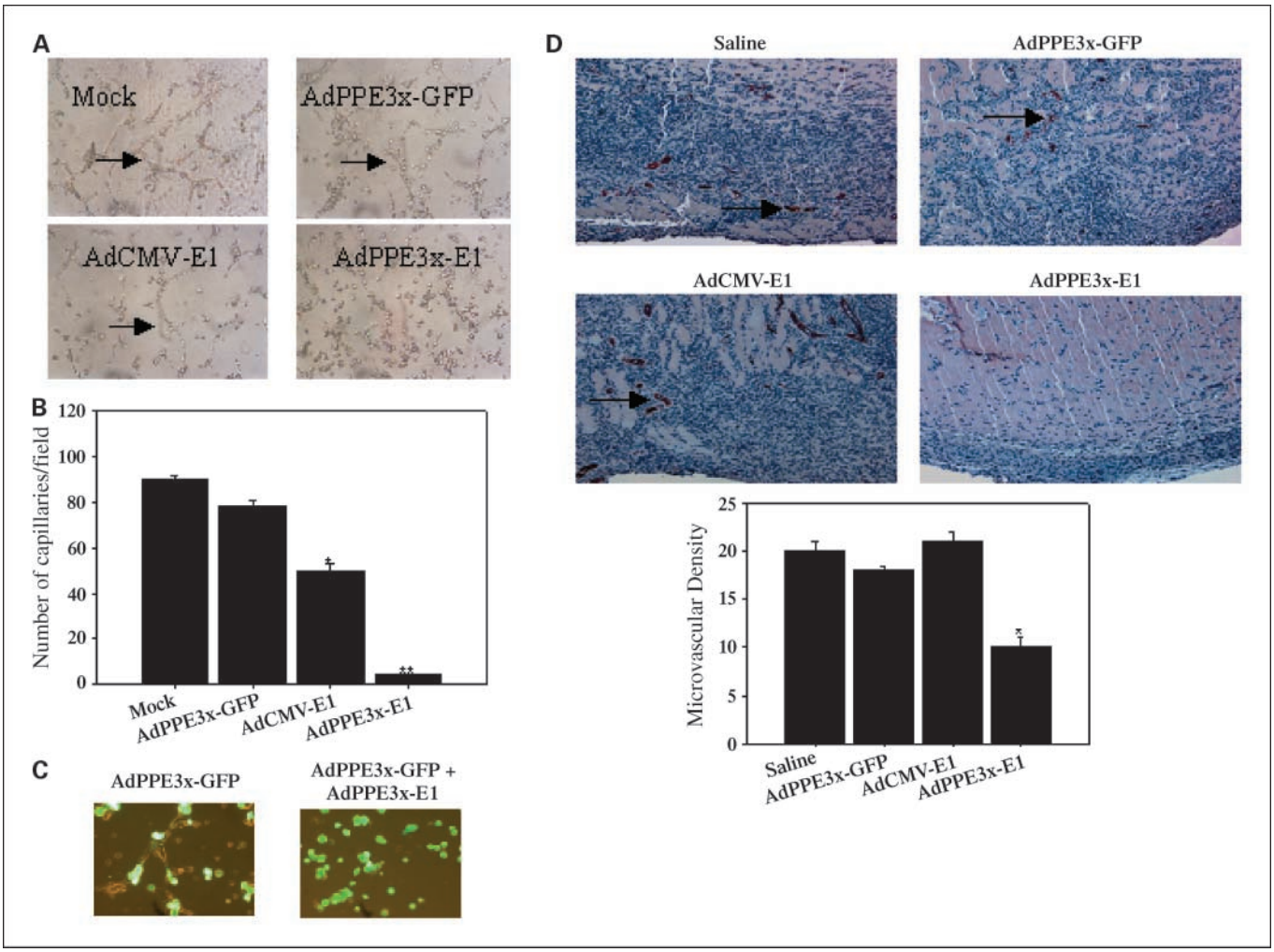

Fig. 3. AdPPE3x-E1 infection inhibits HUVEC differentiation into capillary-like structures and angiogenesis in Matrigel plugs in cotton rats. HUVEC monolayers on 6-well plates were infected with AdPPE3x-E1, AdCMV-E1, and E1-deleted AdPPE3x-GFP virus at 10 MOI. Twenty-four hours later, the infected cells and uninfected controls were seeded on Matrigel, and the spontaneous formation of capillary tubes was recorded at 8 h after plating (*A*) and quantitated as described (*B*). $^{*}P < 0.05$ compared with AdPPE3x-GFP; $^{**}P < 0.01$ compared to AdCMV-E1. Arrows, capillary-like structures. *C,* HUVEC were coinfected with AdPPE3x-E1 and AdPPE3x-GFP, and the formation of capillary tubes was recorded using fluorescence microscopy. *D,* VWF staining of blood vessels (*arrows*) in Matrigel plugs injected into cotton rats, which were resuspended with the different viruses, and quantitated as described. Values are mean ± SE. $^{*}P < 0.05$ compared with all other treatments.

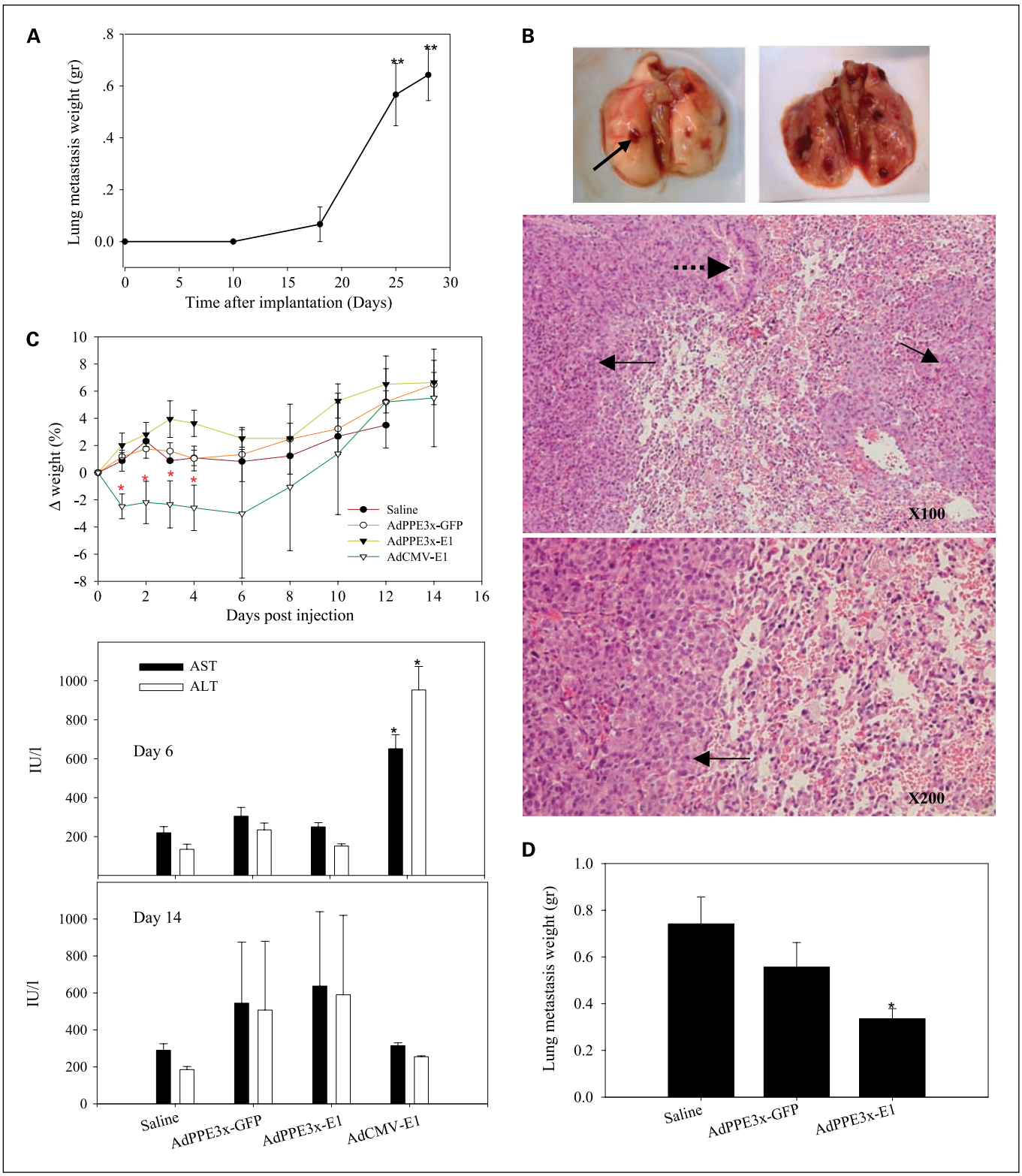

Fig. 4. LCRT lung metastasis model. LCRT cells were injected s.c. on day 0. *A,* lung metastasis weight was calculated by subtraction of the average normal wet organ weight (400 mg) from each tumor-bearing organ. Each bar represents the mean ± SE. n = 2, 3, 3, and 7 for days 10, 18, 25, and 28, respectively. $^{**}P < 0.05$ versus weights on days 10 and 18. *B,* representative lung surfaces of rats on days 25 (*left upper panel*) and 28 (*right upper panel*) following s.c. injection. Arrow, metastasis on lung surface. H&E staining (*two lower panels*) of a lung section that was taken from a rat on day 25, showing normal lung between metastases (arrows, areas of metastases; dashed arrow, bronchiole. ×100, ×200). *C,* groups of three cotton rats were injected systemically with 1×10^{11} particles of AdPPE3x-E1, AdCMV-E1, and AdPPE3x-GFP or saline control. Body weights of animals were assessed every other day for 14 d following i.v. injection. Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected in plasma samples 6 and 14 d following injection. $^{*}P < 0.05$ Vs. all other treatments. *D,* in another experiment, LCRT lung metastases were created in female cotton rats, and AdPPE3x-E1, AdPPE3x-GFP, or saline was injected systemically. Weight due to metastasis burden was calculated as described above. Each bar represents the mean ± SE (n = 8). $^{*}P < 0.05$ versus saline group, based on two-way ANOVA.

compared with metastasis weight at 18 days (Fig. 4A). Macrometastases were absent at 18 days, but were observed beginning at 25 days. Approximately 10 to 15 reddish or white nodules of 1 to 3 mm in maximal dimension on each lung were found. The nodules were soft to hard, and most were in the lung parenchyma. No particular lung lobule was preferred, and the distribution pattern was scattered. Replacement of the lung tissues with lung-tumor deposits was observed at 28 days (Fig. 4B). Histopathologic analysis revealed neoplastic nodules that were composed of round to spindle cells. Lung micrometastases were also observed along bronchioles' walls and blood vessels (Fig. 4B, lower panels). Micrometastases were identified from day 18 following s.c. injection, and by day 28 all animals had developed extensive lung metastases.

Preliminary toxicity tests, in which normal cotton rats were injected with AdCMV-E1, AdPPE3x-E1, AdPPE3x-GFP, or saline, showed hepatic toxicity in the AdCMV-E1–treated rats, as 6 days after i.v. injection of AdCMV-E1 the levels of plasma transaminases of the treated rats increased 4- to 5-fold, compared with AdPPE3x-E1–treated, AdPPE3x-GFP–treated, or saline-treated rats (Fig. 4C, second panel). A mild but significant 3% decrease in body weight was observed in the AdCMV-E1–treated group (Fig. 4C, upper panel), and histopathologic analysis revealed hepatitis, confirming these results. We therefore excluded AdCMV-E1 control from the subsequent *in vivo* experiment. However, it is important to notice that 6 days following infection, the average weight of the AdCMV-E1–treated group started to catch up with the rest of the groups (Fig. 4C, upper panel) and plasma transaminases of the AdCMV-E1–treated group decreased to similar levels of the saline-treated group by 14 days postinfection (Fig. 4C, lower panel).

To evaluate the effect of AdPPE3x-E1 on lung metastases, 15 days following s.c. injection of LCRT cells into the flanks of cotton rats, the rats were injected systemically with AdPPE3x-E1, AdPPE3x-GFP, or saline. The vectors were injected 15 days following s.c. injection of LCRT cells, based on the preliminary experiment, in which micrometastases were observed on day 18, and the average survival rate was evaluated at 28 days after the injection of LCRT cells. After two of the control, saline-injected rats died of metastasis (day 23), all rats were sacrificed. A significant difference was found in the lung metastases weight, which was decreased in the AdPPE3x-E1–treated group by 55% compared with the saline-treated group ($P < 0.05$; Fig. 4D). Vascular density in lung metastases was tested by immunohistochemistry with an anti-VWF antibody. However, only a few blood vessels were found in the metastases, and thus no significant difference in vascular density was found between the different treatments (data not shown). The results suggest that an antimetastasis effect could be induced by the systemic administration of AdPPE3x-E1 with no apparent toxicity.

AdPPE3x-E1 viral titer was increased in lung metastases. To evaluate the viral titer of AdPPE3x-E1 in different organs after systemic administration to cotton rats bearing lung metastases, DNA was extracted from the lungs and livers of the sacrificed rats. Viral DNA was detected by quantitative PCR amplification of the adenoviral *E4* sequence.

AdPPE3x-E1 viral titer in the lung metastases was increased 3- to 5-fold, compared with AdPPE3x-GFP ($P < 0.05$; Fig. 5). No significant increase was observed in the livers, when compared with AdPPE3x-GFP. The results suggest that the antimetastatic effect of AdPPE3x-E1 was due to enhanced replication.

Discussion

In the present work, AdPPE3x-E1, an angiogenesis-transcriptionally targeted adenovirus, was developed. It was shown to preferentially replicate, spread, and lyse endothelial cells *in vitro*, while inducing only minimal cytotoxicity in non-endothelial cells. The *in vivo* results showed the ability of AdPPE3x-E1 to reduce lung metastases burden in cotton rats by 55% when compared with saline-treated rats. Although most of the previously developed CRAds were tested after local administration (i.e., intratumor; refs. 20, 21), we show here an antimetastases effect following systemic administration, by using a CRAd that is transcriptionally targeted to the angiogenic endothelium.

AdPPE3x-E1 targets the expression of the adenoviral *E1A* gene preferentially to dividing endothelial cells. The specific expression is achieved by cloning the adenoviral *E1A* gene under the control of the PPE1-3x promoter, which was previously shown to be highly specific to angiogenic endothelial cells (30, 31), both *in vitro* and *in vivo*, in lung metastases.

However, concerns about the leakiness of the promoters that control the *E1A* gene rose (38), as this leakiness may cause the replication of the CRAd in cells other than the ones targeted and, thus, can damage organs. In previous research (39), it was shown that the transcription factor p300 is essential for the positive regulation of the transcription of *ET-1* in endothelial cells. The availability of p300 is known to be reduced by cotransfection of the adenoviral *E1A* gene, that is, the expression of the *E1A* gene reduces p300 availability and thus

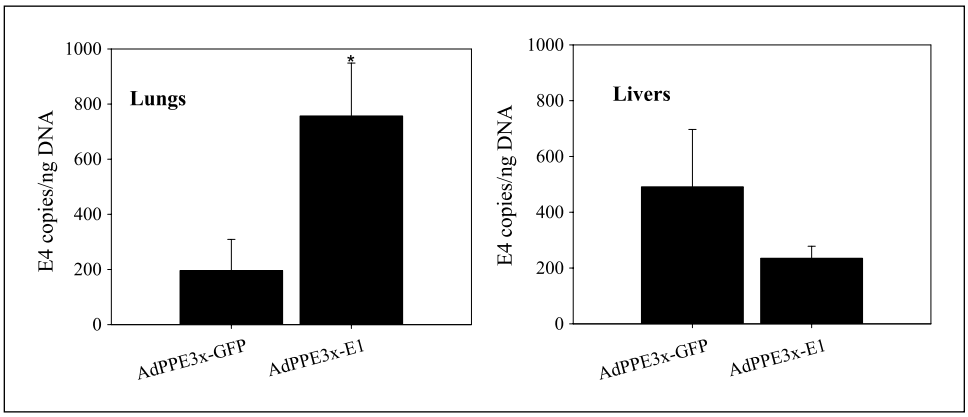

Fig. 5. Enhanced replication of AdPPE3x-E1 in lung metastases following systemic administration to cotton rats transplanted with LCRT tumor cells. *A*, DNA was extracted from lung metastases (*$P < 0.05$ compared with AdPPE3x-GFP) and (*B*) livers, 8 d postinfection. Viral DNA was detected by quantitative PCR amplification of the adenoviral *E4* sequence. The background values (saline-injected rats) were subtracted from the data.

also reduces *PPE-1* transcription. Therefore, there is an inherent "break" in AdPPE3x-E1 that prevents the aforementioned leakiness—expression of *E1A* will reduce its own expression, because *E1A* is controlled by PPE1-3x in AdPPE3x-E1, thereby limiting its levels. Only in cells that express high levels of ET-1, such as angiogenic endothelial cells, will there be enough E1A to support replication. Indeed, AdPPE3x-E1 proved to replicate preferentially in endothelial cells.

We used AdCMV-E1 as a nonspecific replicating adenovector control. Although AdCMV-E1 replicates in endothelial cells and induces a cytopathic effect as efficiently as AdPPE3x-E1 *in vitro*, the reduction that was induced by AdCMV-E1 in the formation of capillary-like structures on Matrigel *in vitro* and *in vivo* was lower than that induced by AdPPE3x-E1. The difference may result from the enhanced transcription induced by the PPE-1 promoter in angiogenesis-like conditions when compared with the CMV promoter, as was previously shown in our lab (30, 31) *in vitro* in proliferating endothelial cells and *in vivo* in lung metastases.

AdPPE3x-E1 was also tested in an *in vivo* cotton rat model. We have shown a reduction in vascular density in the *in vivo* Matrigel model and a reduction in lung metastases burden. To show that the effect of AdPPE3x-E1 on metastases was mediated via an antiangiogenic effect, we also tested vascular density in the lung metastases by immunohistochemistry with an anti-VWF antibody. However, only a few blood vessels were stained in the lungs, and thus no significant difference in vascular density could be shown (data not shown). These results may indicate that future experiments should be conducted with different kinetics, to give more time for angiogenesis to develop in the lung metastases, thus enabling observation of a significant difference in vascular density. However, the fact that AdPPE3x-E1 replicates poorly in LCRT cells (Fig. 1) implies that its *in vivo* effect on metastases is not mediated by replication in the tumor cells. Moreover, vascular density was significantly reduced *in vivo* in the Matrigel model (Fig. 3D), showing the antiangiogenic effect of AdPPE3x-E1 *in vivo*.

As mentioned above, the cotton rat tumor model has several advantages over immunodeficient mice bearing xenograft human tumors, that stem from the permissiveness of the cotton rat to adenoviral replication. However, previous results from Karoly et al. (22) showed that adenovirus replication in cells from cotton rat origin was reduced 100-fold when compared with human cells. Moreover, when LCRT s.c. tumors in cotton rats were injected intratumorally with adenovirus, a continuous decrease in viral particles was observed until no viruses were detected in the tumor at 10 days following injection (22). These results imply that cotton rats are only semipermissive for adenoviruses. Jogler et al. (40) have recently found out that porcine cells support productive replication of human adenovirus type 5 nearly as efficiently as human cells, whereas the release of infectious virus by cells from other animal species tested was diminished by several orders of magnitude. Thus, future experiments, with the required facilities to produce large amounts of viruses for the injection of CRAds into swine, will enable improvement of the current model.

Although the capacity of the virus to inhibit metastases is of principal importance, viral toxicity following systemic administration is also a major concern. Hepatic toxicity was shown in the AdCMV-E1–treated rats (Fig. 4C) only, without any significant toxicity in the AdPPE3x-E1–treated rats. However, given that the adenovirus replicates 100 times more in humans than in cotton rats (22), its toxicity might also be increased in humans, especially in the liver which is the main organ that is infected by adenoviral systemic administration (41). Notwithstanding this concern, the liver toxicity induced by AdCMV-E1 was transient (Fig. 4C), and given that AdPPE3x-E1 replicates 60 times slower than AdCMV-E1 in hepatic cells (Fig. 1), it is reasonable to assume that the liver toxicity of AdPPE3x-E1 in humans will also be transient. AdPPE3x-E1 might also target other processes in which angiogenesis is important, like wound healing. Thus, careful titration of the virus is important for reducing its toxicity to minimum.

We have presented, for the first time, the effect of an angiogenesis-transcriptionally targeted CRAd *in vivo*, in an immunocompetent model. It was shown to reduce lung metastases burden by 50%. Keeping in mind that an adenovirus replicates 100 times less in cotton rats than in humans (22), this vector may have important applications in the clinical setting.

Disclosure of Potential Conflicts of Interest

D. Harats is employed by, has received a commercial research grant from, and has an ownership interest in, Vascular Biogenics, Ltd. I. Barshack and A. Shaish are consultants for Vascular Biogenetics, Ltd.

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Clin Cancer Res 2009;15:1664-1673.